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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The recent development of fluorescently–tagged deoxyglucose analog (2-NBDG) has attracted the attention of researchers and clinicians alike for its potential use as a tool in detecting cancer. Although the use of 2-NBDG imaging for cancer diagnosis has been explored in several other organ sites prior to this project, the potential applications of this technique to early lung cancer detection had not been explored. The objective of this study was to monitor both qualitatively and quantitatively 2-NBDG uptake in lung cancer cells and normal cells using fluorescence imaging techniques. Initial results were promising, and this work represents an important first step towards establishing the use of 2-NBDG as a contrast modality for applications in early lung cancer diagnosis.

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molecular imaging, optical imaging

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Table of Contents

Introduction

There is a compelling need for new methods for early detection of lung cancer. This project is based on a belief that current technologies do not adequately address this need. PET imaging has a useful role in non-small cell lung cancer detection but the spatial resolution of the method provides a lower bound on detectable tumor sizes. Fluorescence bronchoscopy has proved a valuable adjunct to white light bronchoscopy images for some clinical applications but sensitivity and specificity concerns have limited widespread adoption of the approach. The project focuses on a method which combines the sensitivity to glucose metabolism of PET imaging with the higher resolution – and thus, potential ability to detect earlier disease states – of optical approaches. The method adds to the capabilities of bronchoscopy without substantially increasing cost. Although there has been no work evaluating the use of 2-NBDG for lung cancer applications, prior work in the literature has demonstrated the value of such an approach for oral cancer applications and motivates this project. A basic science arm of the study evaluated the potential of 2-NBDG for early lung cancer detection *in vitro* quantifying fluorescence signals and their variability after 2-NBDG application in a series of normal and lung cancer cell lines as a function of concentration and time. An imaging arm of the study focused on wide-field macroscopic and sub-cellular resolution microscopic fluorescent images after 2-NBDG application simulating images which would be obtainable via bronchoscopy. In the report that follows, both pieces of the study are described together. As we further investigated prior use of 2-NBDG, it became evident that the literature was not fully consistent as to the success achieved using this approach. While we had initially planned to use both cell systems and 3D RAFT culture models, this one year pilot study focused on 2D systems as we needed to more thoroughly assess the potential use of the method in light of some of the literature we found indicating negative results using ^{18}FDG in the same model. While we did not have time perform the experiments in RAFT cultures yet, we plan to do so in the future. Imaging results are integrated together with the quantitative studies in the report that follows.

Progress Report Body

Introduction

Lung cancer is the leading cause of cancer mortality in the United States for men and women. An estimated 200,000 new cases and 159,000 deaths were expected in 2013. For over three decades, fluorescence spectroscopy has been utilized to discriminate normal from pathological conditions for various organs. In particular, advanced research in fluorescence-tagged glucose analogues has attracted the attention of clinicians and biomedical researchers (1, 2, 3, 4). Glucose is well known to promote the energy needed for cell growth; therefore, fast-growing cancer cells require more glucose than cells growing under normal condition. Glucose uptake has been tracked in various cells and tissues, and it has also been found that glucose consumption is markedly increased in cancer cells when compared to normal cells. In fact, one of the biochemical markers in tumor malignancy is enhanced tumor glycolysis, primarily based on the overexpression of glucose transporters (Gluts) and the increased activity of mitochondria-bound hexokinases in tumors (5, 6). Since 1976, 2-(fluorine-18)-(fluoro-2-deoxyglucose) (^{18}FDG) has been utilized for the noninvasive monitoring of glucose utilization (7), and ^{18}FDG-PET is routinely used for brain mapping or cancer diagnosis and staging (8). The uptake mechanism and biochemical pathways of ^{18}FDG have been studied *in vitro* and *in vivo*, and glucose transport through the cell membrane via Gluts has been reported as an important factor in the increase of FDG uptake in malignant tumors (5). In particular, various researchers reported that ^{18}FDG uptake correlated well with overexpression of Glut-1 and/or Glut-3 in non-small-cell lung cancer (NSCLC); however, a few researchers have reported contradictory findings (5, 8). Consequently, the major factor influencing FDG uptake in NSCLC remains controversial.

2-N(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) is a fluorescence analogue that follows a pathway of uptake and metabolism similar to that of FDG and accumulates preferentially in malignant cells or tissues. However, the use of fluorescently labeled deoxyglucose has several advantages compared with FDG. More specifically, the fluorescence analogue is superior to isotope analogues in terms of temporal and spatial resolution

(9). 2-NBDG was initially used to image and measure signaling pathway-simulated glucose uptake in single cells from the pancreas, and the method was also followed to validate novel antidiabetic compounds with similar effects, such as the hormone insulin (9). Later, the fluorescence analogue 2-NBDG was primarily used to directly monitor glucose uptake by living cells (1, 10). In addition to its use as a topical contrast reagent for the detection of neoplasia, it can be used in real-time confocal, high-resolution, or wide field fluorescence microscopy, as well as in flow cytometry (2, 4, 10). O'Neil et al. have measured the uptake kinetics of 2-NBDG in cancer cell lines, including MCF-7 breast cancer and HepG2 liver cancer, as well as a normal M-1 epithelial cell line, demonstrating rapid uptake and retention of 2-NBDG in the cancer cells (1). Millon et al. monitored 2-NBDG uptake in breast normal and cancer cells and also studied the influence of Glut-1 in normal and breast cancer cells (10). Nitin et al. and Langsner et al. have demonstrated the use of 2-NBDG to enhance the fluorescence imaging of oral neoplasia and breast cancer lesion, respectively (2, 4). Sheth et al. used 2-NBDG as a molecular beacon probe to monitor glucose utilization, both *in vitro* and *in vivo* (3). Recently, Peng et al. noted the increased uptake of 2-NBDG in circulating breast cancer cells in blood samples from tumor-bearing mice, but not in the blood samples from normal control mice (11). However, since no substantial studies have been carried out to monitor 2-NBDG uptake in lung cells and tissues, the first objective of this study was to monitor 2-NBDG uptake in small-cell lung cancer cells and normal fibroblast lung cells by fluorescence both quantitatively (Aim/Task 1) and through sub-cellular and widefield (Aim/Task 2) imaging. While DoD funds were not used to purchase cells lines, it is still worth noting minor changes were made in the cells used as a result of our literature review at the beginning of the project.

Materials and Methods

Cell preparation for 2-NBDG uptake

Human lung normal (WI38) and cancer (A549) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). WI38 and A549 were cultured in Eagle's minimum essential medium (EMEM) and F-12K medium, respectively. Cells were maintained in an incubator at 37°C. For fluorescence microscopy study, cells were plated at 100,000 cells/ml in 12-well plates and used at subconfluence after 48 hours preincubation. After 48 hrs, with cells in their log growth phase, the medium was removed, and PBS with 1% bovine serum albumin

(BSA) was added to the wells for 20 minutes at 37°C to starve the cells. Cell medium alone was used as a control. The PBS-BSA was then aspirated, and 100, 200 and 500 µm concentration of 2-NBDG was added to the wells for 20 minutes at 37°C in darkness. The experiment was performed two times, with two wells used for each concentration. The 2-NBDG was then aspirated and rinsed twice with cold PBS to remove any nonmetabolized 2-NBDG.

Fluorescence Imaging

Immediately after the cells were washed, they were placed for fluorescence imaging. The cells were excited with filtered light at 450-490nm, and the emission was collected at 500-550nm using a CCD camera with 500 milliseconds (ms) exposure time. Fluorescence microscopy images for two samples for each concentration of 2-NBDG-treated cells and two positive control and negative control samples were captured for 10X, 20X and 40X magnification.

Flow cytometry study

In order to quantify 2-NBDG uptake in normal and cancer lung cells for flow cytometry, we are waiting for normal lung cells to grow to confluence. At that point, we can compare the quantity of glucose between normal and cancer cells by 2-NBDG uptake using flow cytometry. For the present experiment, we measured the concentration-dependent fluorescence intensity of 2-NBDG-labeled A549 cells against unlabeled cells. To accomplish this, A549 cells were plated at 100,000 cells/ml in 24-well plates and used at subconfluence after 48 hours pre-incubation. To perform this experiment, all cell culture media were removed, 500 µl trypsin was added, and cells were left in an incubator at 37°C for 5 minutes. Next, 500µl of 2-NBDG was added to cells in concentrations of 5, 100 and 200µm, with 500µl of medium for control, and the cells were left to incubate for 20 minutes, then pelleted by centrifugation, resuspended with fixation buffer, and, finally, analyzed by flow cytometry. For each measurement, data from 20000 single-cell events were collected using flow cytometry.

Results and Discussion

1. Monitoring 2-NBDG uptake on normal and cancer lung cells

1A. Fluorescence microscopy imaging for 2-NBDG uptake on normal and cancer lung cells

Figure 1-4 represent the images of both normal and cancer lung cells before and after treatment of 2-NBDG with 100, 200 and 500 µm concentrations at 10x, 20x and 40x magnification. In the absence of 2-NBDG, background fluorescence was not observed in either normal (Figure 1) or cancer cells (Figure 2). However, the 2-NBDG-treated cancer cells clearly showed a brighter fluorescence signal than the 2-NBDG-treated normal cells indicating the increased uptake of 2-NBDG in lung cancer cells compared to normal lung cells (Figures 3 and 4). Fluorescence images for the lower magnification (10x) show brighter fluorescence than those at the higher magnification (20x and 40x). For the 2-NBDG treatments at higher concentration (500µM), normal cells also show considerable fluorescence when compared with cancer cells. However, for lower concentrations (100 and 200µm), cancer cells show distinctly brighter fluorescence than normal cells. Especially, fluorescence images of cancer cells treated with a 100µm concentration of 2-NBDG are distinctly brighter than those reported in an earlier study by Park et al. (6). They imaged 10^4 A549 cancer cells (compared with our cell concentration of 10^5) treated with a concentration of 125 µm 2-NBDG at an exposure time of 11000 ms. They did not detect 2-NBDG fluorescence for 500ms of exposure time, whereas we observed distinct fluorescence for A549 cancer cells treated with 100µm 2-NBDG-treated cancer cells for the same exposure time, but little fluorescence for normal lung cells. Hence, our study clearly demonstrated fluorescence increase after the treatment of 2-NBDG in cancer cells compared to normal cells.

Figure 1. Fluorescence images of unlabeled [normal at 10X (1A), 20 X (1B), 40X (1C) magnification] and [cancer at 10X (2A), 20X (2B), 40X (2C) magnification] lung cells.

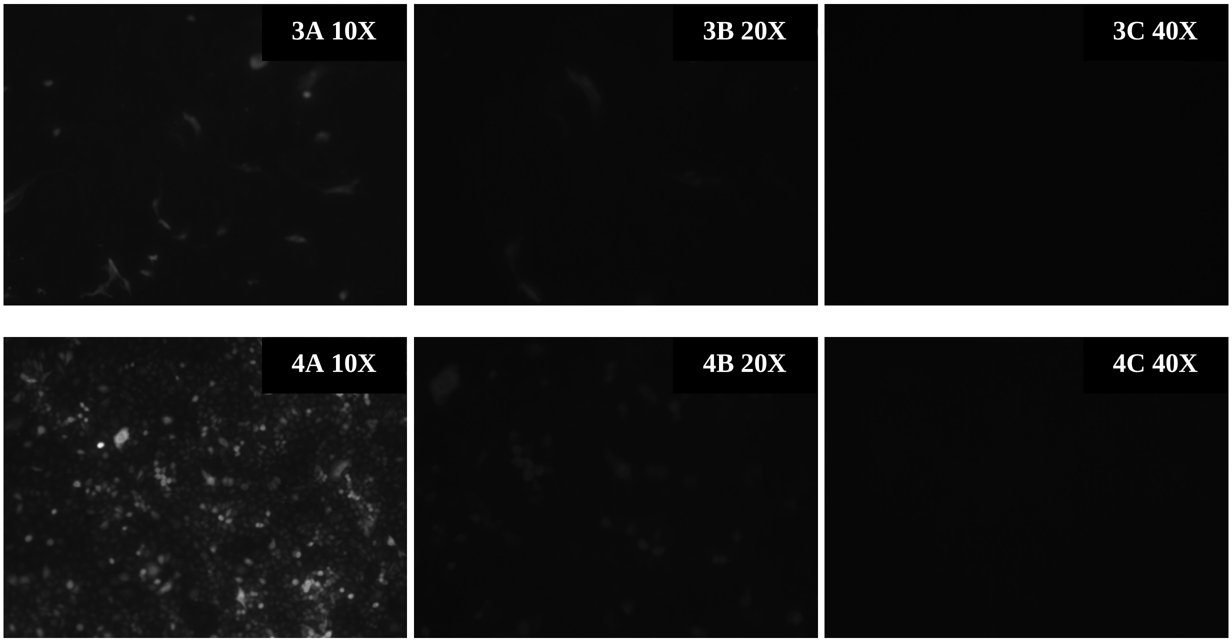

Figure 2. Fluorescence images of 100 µm 2-NBDG-treated [normal at 10X (3A), 20 X (3B), 40X (3C) magnification] and [cancer at 10X (4A), 20X (4B), 40X (4C) magnification] lung cells.

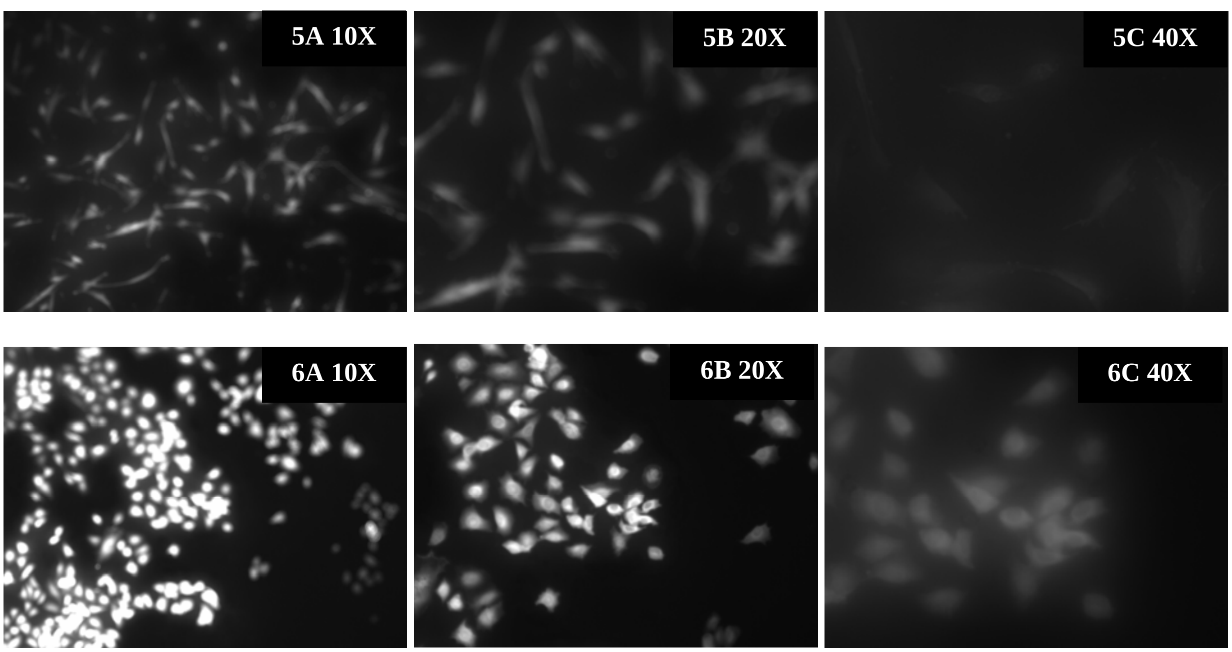

Figure 3. Fluorescence images of 200 µm 2-NBDG-treated [normal at 10X (5A), 20 X (5B), 40X (5C) magnification] and [cancer at 10X (6A), 20X (6B), 40X (6C) magnification] lung cells.

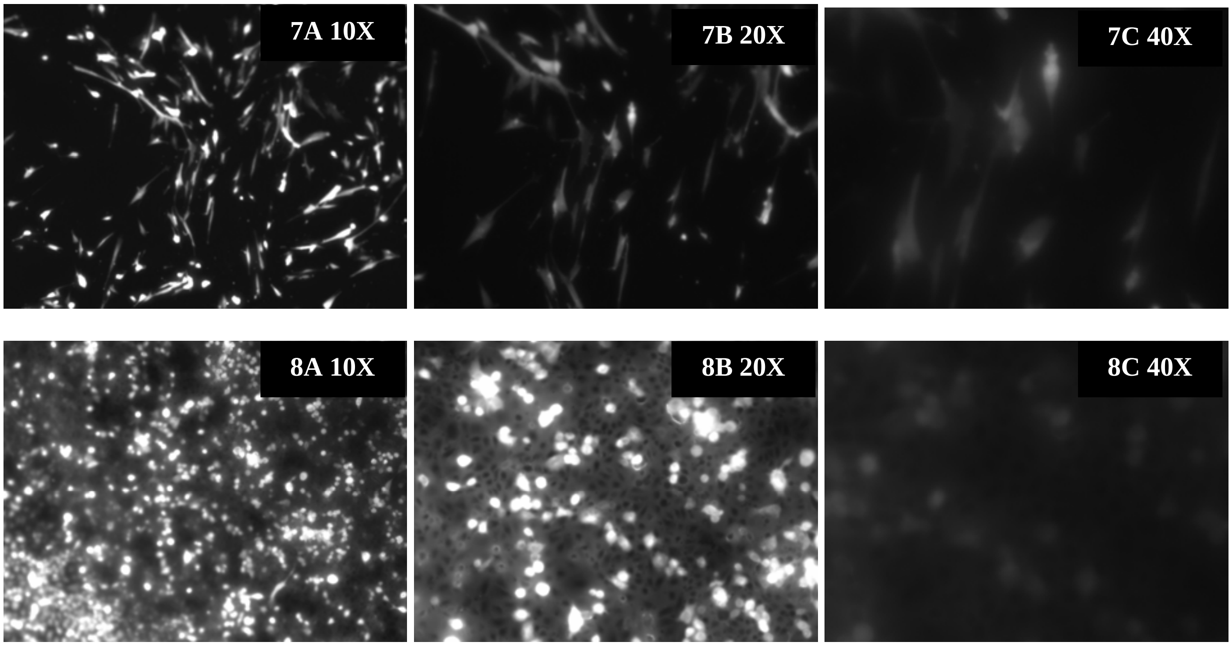

Figure 4. Fluorescence images of 500 µm 2-NBDG-treated [normal at 10X (7A), 20 X (7B), 40X (7C) magnification] and [cancer at 10X (8A), 20X (8B), 40X (8C) magnification] lung cells.

An earlier study reported that malignant cells show increased glucose uptake *in vitro* and *in vivo* (5). This process is thought to be mediated by glucose transporters (Gluts), the expression and activity of which are regulated by oncogenes and growth factors. These glucose transporters have different distribution in human organs. In this context, several researchers studied the effect of Gluts in various organs, including Non-small-cell lung carcinoma (NSCLC). Younes et al. showed that Glut-1 and Glut-3 were expressed in Stage I NSCLC cells by immunohistochemistry, and the study showed that 83% and 21% of NSCLC cases were positive for Glut-1 and Glut-3, respectively (5). Many other researchers studied the influence of various types of glucose uptake in NSCLC, in particular FDG uptake in NSCLC (8). However, only a few have reported FDG uptake in the context of Glut-1 and Glut-3 expression, or the correlation between FDG uptake and the overexpression of Glut-1/Glut-3 in NSCLC. It should be noted that other investigators have reported contradictory findings (12). Thus, glucose uptake in NSCLC and the distribution of Glut-1/Glut-3 remain controversial.

However, over the past decade, many researchers have studied the effect of 2-NBDG uptake in various cells and tissues. Also, many others have developed mouse tumor models to monitor the effect of glucose uptake (3, 11). However, no substantial studies have attempted to monitor the 2-NBDG uptake mechanism in human lung cells or tissues. Park et al. monitored 2-NBDG uptake in A549 cells, but they did not observe any significant results (6). In this context, the present study aimed to monitor 2-NBDG uptake in human A549 lung cancer cells and WI38 fibroblast normal lung cells by fluorescence microscopy imaging. Our preliminary study showed higher fluorescence for the cancer cells when compared to normal cells, indicating the higher uptake of 2-NBDG in cancer cells. This study represents a promising initial step towards developing 2-NBDG as a modality for application in diagnostically imaging lung cells and

tissues. However, further study is needed to quantify glucose uptake between normal and cancer lung cells to strengthen this study.

1B. Quantification of 2-NBDG uptake on normal and cancer lung cells by flow cytometry

In order to quantify 2-NBDG uptake in normal and cancer lung cells for flow cytometry, we are waiting for normal lung cells to grow to confluence. At that point, we can compare the quantity of glucose between normal and cancer cells by 2-NBDG uptake using flow cytometry. For the present experiment, we measured the concentration-dependent fluorescence intensity of 2-NBDG-labeled A549 cells against unlabeled cells. For each measurement, data from 20000 single-cell events were collected using flow cytometry. Figure 5 and 6 show the fluorescence intensity of 2-NBDG-treated lung cancer cells (A549) at 0, 5, 100 and 200 µm concentrations. The results showed the increased fluorescence signal for 2-NBDG-treated cells with respect to the increasing concentration of 2-NBDG and showed overall higher fluorescence intensity than that of unlabeled lung cancer cells.

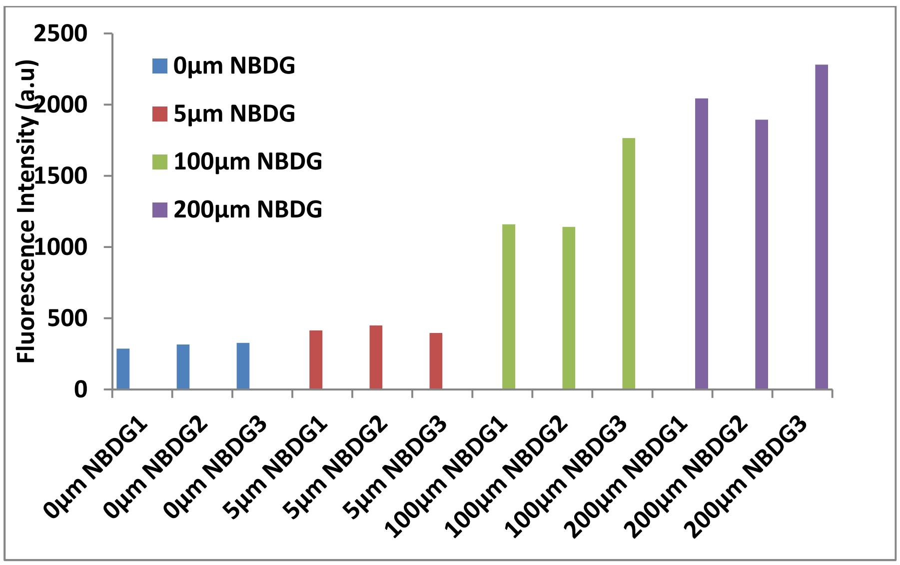

Figure 5. Fluorescence intensity changes of 2-NBDG- labeled and unlabeled lung cancer cells.

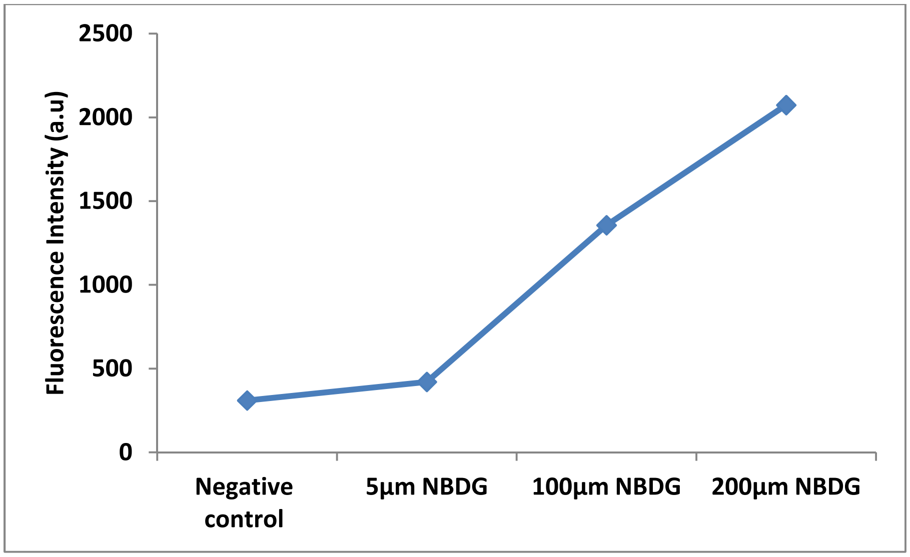

Figure 6. Average fluorescence intensity changes of 2-NBDG-labeled and unlabeled lung cancer cells.

Key Research Accomplishments

- This study clearly showed increased uptake of 2-NBDG in lung cancer cells (NSCLC) as compared to normal lung cells.

- This study represents a promising initial step towards developing 2-NBDG as a modality for the lung diagnostic imaging, and provides additional evidence to support increased glucose uptake in NSCLC. Increased glucose uptake in NSCLC has been a disputed area in the literature with published studies on results based on FDG containing contradictory findings. We believe that using a different mechanism to look at glucose uptake, as we did in this study, will substantively contribute to the literature in this area.

Reportable Outcomes

None to date. A paper is planned once additional experiments are completed. Because our results are not able to be fully consistent with prior literature (as the literature itself does not present a consistent view), we are doing more extensive experiments prior to publication.

Conclusion

The recent advances in the development of molecular-based approaches have the potential to improve both the detection of cancer. In particular, the recent development of a fluorescence–tagged glucose bioprobe (2-NBDG) has attracted the attention of researchers and clinicians alike for its utility as a tool in detecting cancer. A PubMed search indicates few articles (54) reported for both 2-NBDG and 6-NBDG from 2005 to 2010 in comparison to an abundance of articles reporting on 2-NBDG applications alone in 2011 (21), 2012 (19) and 2013 (13) (Figure 7). These statistics indicate the rapid development of 2-NBDG as a method of monitoring glycolysis in cancer cells. While most of these studies have focused on the use of 2-NBDG imaging for cancer diagnosis in several organ sites, no comparable studies had been carried out in a lung model. Therefore, the present study aimed to (1) monitor 2-NBDG uptake in cancerous and normal fibroblast lung cells and characterize such uptake by measuring changes in metabolic activity of individual NSCLC cells, as determined by both fluorescence imaging and flow cytometry. This study represents a promising initial step towards developing 2-NBDG as a modality for the diagnostic imaging of lung cells and tissues.

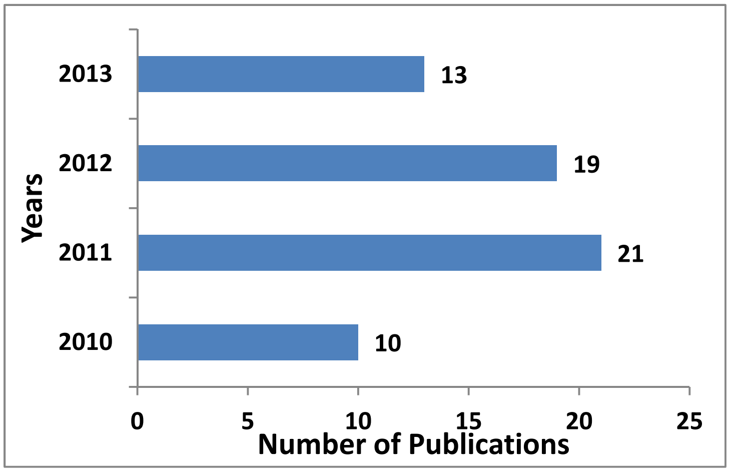

Figure 7. Number of publications for 2-NBDG from 2010 to 2013

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Vengadesan Nammalvar

Bibliography

No publications/abstracts to date.

Abbreviations

2 NBDG	2-N(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose
^{18}FDG	2-(fluorine-18)-(fluoro-2-deoxyglucose)
NSCLC	non small cell lung cancer